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P. WYREMBEK[1], K. SZCZURASZEK[1], M.D. MAJEWSKA[3,4], J.W. MOZRZYMAS[1,2]

INTERMINGLED MODULATORY AND NEUROTOXIC EFFECTS OF THIMEROSAL AND MERCURIC IONS ON ELECTROPHYSIOLOGICAL RESPONSES TO GABA AND NMDA IN HIPPOCAMPAL NEURONS

[1]Laboratory of Neuroscience, Department of Biophysics, Wroclaw Medical University, Wroclaw, Poland; [2]Department of Animal Physiology, Institute of Zoology, Wroclaw University, Wroclaw, Poland; [3]Marie Curie Chairs Program at the Department of Pharmacology and Physiology of the Nervous System, Institute of Psychiatry and Neurology, Warsaw, Poland; [4]Department of Biology and Environmental Science, University of Cardinal Stefan Wyszynski, Warsaw, Poland

The organomercurial, thimerosal, is at the center of medical controversy as a suspected factor contributing to neurodevelopmental disorders in children. Many neurotoxic effects of thimerosal have been described, but its interaction with principal excitatory and inhibitory neurotransmiter systems is not known. We examined, using electrophysiological recordings, thimerosal effects on GABA and NMDA-evoked currents in cultured hippocampal neurons. After brief (3 to 10 min) exposure to thimerosal at concentrations up to 100 μM, there was no significant effect on GABA or NMDA-evoked currents. However, following exposure for 60-90 min to 1 or 10 μM thimerosal, there was a significant decrease in NMDA-induced currents ($p<0.05$) and GABAergic currents ($p<0.05$). Thimerosal was also neurotoxic, damaging a significant proportion of neurons after 60-90 min exposure; recordings were always conducted in the healthiest looking neurons. Mercuric chloride, at concentrations 1 μM and above, was even more toxic, killing a large proportion of cells after just a few minutes of exposure. Recordings from a few sturdy cells revealed that micromolar mercuric chloride markedly potentiated the GABAergic currents ($p<0.05$), but reduced NMDA-evoked currents ($p<0.05$). The results reveal complex interactions of thimerosal and mercuric ions with the $GABA_A$ and NMDA receptors. Mercuric chloride act rapidly, decreasing electrophysiological responses to NMDA but enhancing responses to GABA, while thimerosal works slowly, reducing both NMDA and GABA responses. The neurotoxic effects of both mercurials are interwoven with their modulatory actions on $GABA_A$ and NMDA receptors, which most likely involve binding to these macromolecules.

Key words: *$GABA_A$ receptors, neurotoxicity, NMDA receptors, patch-clamp, thimerosal, mercuric ions, hippocampal neurons*

INTRODUCTION

Thimerosal (THIM), an organomercurial containing approximately 49% of mercury by weight, has been added for decades to medicinal products, including pediatric vaccines, without being sufficiently tested for its safety. This is surprising in view of the fact that all mercurials are highly toxic, particularly to developing organisms. In the past decade concerns emerged about the possibility that THIM from vaccines might contribute to certain neurodevelopmental disorders in children, which prompted its recent removal from most pediatric vaccines in the Western countries (7, 19). Unfortunately, it is still added to pediatric vaccines in less developed countries, including Poland, potentially damaging the health of children.

THIM is metabolized in the body to ethyl mercury (EtHg) and subsequently to inorganic mercury forms, which accumulate in tissues of vital organs, including the brain (22). Information about neurochemical and neurotoxic effects of THIM is still limited, but the existing data indicate that in pharmacodynamics and toxicity THIM/EtHg does not differ significantly from methyl mercury (MeHg), which has been studied more extensively, although these compounds differ somewhat in pharmacokinetics (8).

Several studies documented that the neurotoxic effects of mercurials involve glutamate-mediated excitotoxicty, due to their ability to inhibit uptake of glutamate in astrocytes, resulting in an increase of the extracellular level of this excitatory amino acid (1, 4, 14). Excessive synaptic activity of glutamate may lead to excitotoxicity. Mercurials may interact as well with the glutamate receptors. MeHg has been shown to alter gene expression for the NMDA receptors (16) and to inhibit NMDA receptor binding *in vitro* (23), but in electrophysiological recordings both MeHg and $HgCl_2$ were without apparent rapid modulatory effect on the NMDA-induced currents in neurons (25). Equally ambiguous are the effects of mercurials on function of $GABA_A$ receptors. Electrophysiological studies demonstrated that both MeHg and inorganic Hg interact with neuronal $GABA_A$ receptors, albeit in opposite directions, as $HgCl_2$ potentiated the GABAergic currents, whereas MeHg decreased them (11, 20). An *in vivo* study showed an increased number of benzodiazepine receptors in rat brain, three days after acute MeHg administration (9).

There are no published data on interactions of THIM with the $GABA_A$ or NMDA receptors, but some type of interactions may be expected based on similarities of this compound to MeHg. The aim of this study was to compare the effects of micromolar concentrations of THIM and $HgCl_2$ on GABAergic and NMDA induced currents in primary hippocampal cultures. We report complex short- and long term effects of these mercurials on $GABA_A$ and NMDA receptors.

MATERIALS AND METHODS

Cell culture for electrophysiological recordings

Primary cell culture was prepared as previously described by Andjus *et al*. (3, 24). The procedure was performed in accordance with the regulation of the Polish Animal Welfare Act and was approved by the local Ethical Committee (Decision No 18/2010). Briefly, P1-P3 days old Wistar rats were decapitated. Hippocampi were dissected, manually sliced, treated with trypsin, mechanically dissociated and centrifuged twice at 40 g, plated in the Petri dishes and cultured. Experiments were performed on neuronal cells cultured for 10 to 17 days. Experiments were performed on 3 cultures, each prepared from 3-4 pups. No difference were observed with respect to the considered THIM effects within this culture period.

THIM (sodium ethylmercurithiosalicylate) used in electrophysiological experiments was obtained from Sigma. Stock solutions of 10 mM THIM was prepared in water. To check for the effect of mercuric ions on NMDA or GABA-elicited responses, mercuric chloride ($HgCl_2$ Sigma-Aldrich, Mercury (II) chloride, USA) was added to the solutions.

Electrophysiological recordings

Currents were recorded in the whole-cell configuration of the patch-clamp technique using the Axopatch 200B amplifier (Molecular Instruments, Sunnyvale CA, USA) at a holding potential (V_h) of -40 mV. The intrapipette solution contained (in mM) CsCl 137, $CaCl_2$ 1, $MgCl_2$ 2, 1,2-bis(2-aminophenoxy) ethane-N,N,N'-tetraacetic acid (BAPTA) 11, ATP 2, HEPES 10 (pH 7.2 with CsOH). For recordings of GABAergic currents, the composition of the standard external solution was (in mM) NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, HEPES 10 (pH 7.2 with NaOH). In the case of NMDA-evoked current responses the following external solution was used: NaCl 137, KCl 5, $CaCl_2$ 2, glucose 20, HEPES 10, glycine 0.1 (pH 7.2 with NaOH). In addition, to block the current component mediated by glycine receptors, 2 μM strychnine was added to the external solution. For the whole-cell recordings, patch pipettes had 2.5-4.0 MΩ when filled with the internal solution. The whole-cell recordings were considered for analysis when the access resistance was below 10 MΩ. The cells in which series resistance showed a clear tendency to increase during recordings were not considered in the analysis. Whole-cell recordings were started at least 3 min after establishing the whole-cell configuration. This time was sufficient to stabilize the recording conditions. To avoid excessive synaptic activity in the whole-cell configuration, TTX (Latoxan, France) at 1 μM concentration was added to the external solution.

The solutions were applied to cells using the RSC-200 multibarrel rapid perfusion system (Bio-Logic, Grenoble, France). With this system, in the whole-cell configuration, the solution exchange around neurons adhering to the bottom occurred within 30-100 ms. Before recording control current responses, cells were washed with normal external solution for at least three minutes. The impact of THIM on GABAergic and NMDA-evoked currents was studied in two protocols designed to test the acute and long-term effects. In the first case, THIM was present both in the washing solution (for at least 3 minutes before agonist application) and in the agonist-containing saline. Thus, before and during agonist application, the tested agent was present at the same concentration. This protocol allowed comparison of the currents recorded in the presence of THIM with controls obtained from the same neuron, therefore the paired test (*t* Student paired test) could be used and the impact of the studied agent was assessed by calculating the relative amplitude values. To test for the long-term THIM effect on GABA or NMDA-evoked currents, coverslips with neurons from the same culture were randomly divided into two equal groups and one group was considered as control and the other was treated for 60-90 min with a selected THIM concentration (1 or 10 μM). The current responses were then recorded as described above for controls and THIM-treated neurons. In the group of THIM-treated neurons, this drug was added immediately after the culture medium was substituted with the external solution. For this reason, respective controls were recorded after 60-90 min incubation of neurons in external saline. For statistical comparison of the control group with group of neurons long-term treated with THIM, the standard unpaired Student's *t* test was used. In a single experiment, performed during one working day, data for only one THIM concentration could be confronted with respective controls. For this reason, control data for each THIM concentration group were collected separately on each experimental day, to assure age uniformity of tested neurons. Since both GABA and NMDA-evoked currents show large cell-to-cell variability, a large number of cells needed to be tested to accurately assess the effect of long-term treatment with THIM. For acquisition and data analysis, pClamp 10.2 software was used (Molecular Device Corporation).

For the analysis of currents, recorded in the whole-cell configuration, the current signals were low-pass filtered at 3 kHz with a Butterworth filter and sampled at 20 kHz using the analog-to-digital converter Digidata 1322A (Molecular Device Corporation) and stored on the computer hard disk.

In the cells treated for 60-90 min with THIM and respective controls, the cell viability was tested with trypan blue probe. To perform this test, cultures were exposed at least two times to trypan blue-containing buffer (0.4% in 0.81% sodium chloride; 0.06% potassium phosphate, dibasic, Trypan Blue solution, Fluka). Each time, after trypan blue treatment, cells were washed with the culture medium. The total number of cells and the number of trypan blue stained neurons were counted in three randomly chosen fields in a coverslip on which neurons were plated (standard optical phase contrast microscope, Nikon Eclipse, TS100 was used for cell visualization). Data are expressed as means ±S.E.M.

All experiments were performed at room temperature 22-24°C.

RESULTS

Acute application of THIM does not affect GABAergic and NMDA-evoked currents

GABAergic currents were elicited in the whole-cell configuration by rapid application of 3 μM GABA at a holding voltage of -40 mV. Typical GABA-evoked response recorded in these conditions is presented in *Fig. 1A*. The effect of THIM on current amplitude was assessed by dividing the current amplitude measured in the presence of this drug by the amplitude value in control conditions measured from the same cell. As shown in *Fig. 1B*, acute application of 100 μM THIM, did not exert any significant effect on current amplitude (relative amplitude 1.07±0.1, $p>0.05$, n=17). At lower THIM

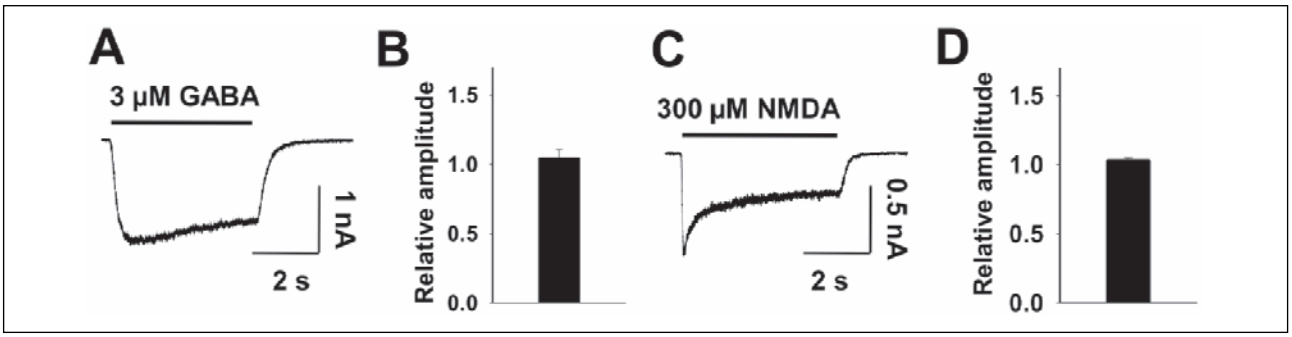

Fig. 1. Acute treatment with thimerosal at concentration 100 μM does not affect the amplitude of either GABA (A,B) or NMDA-evoked (C,D) currents. A, Typical current trace evoked by rapid application of 3 μM GABA at -40 mV. B, Statistics of relative amplitudes of recorded GABAergic currents calculated for THIM at 100 μM ($p>0.05$, n=17). C, Typical current trace evoked by rapid application of 300 μM NMDA at -40 mV. D, Statistics of relative amplitudes of recorded NMDA-evoked currents calculated for 100 μM THIM ($p>0.05$, n=4).

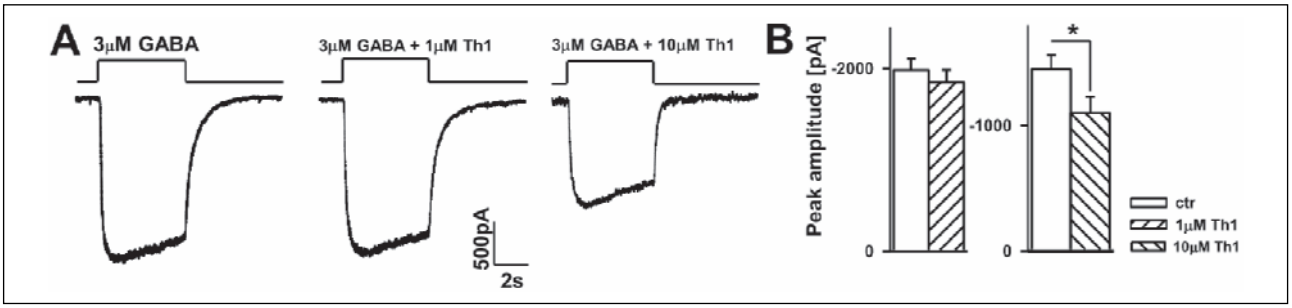

Fig. 2. Prolonged (60-90 min) treatment with THIM affects the amplitude of GABAergic currents. A, typical current responses elicited by rapid application of 3 μM GABA in control conditions (left), in the presence of 1 μM THIM (middle) and in the presence of 10 μM THIM (right). Insets above the traces represent the time of agonist application. B, statistics of THIM effect on current amplitude at 1 μM (left) and at 10 μM (right). White bars represent controls white dashed bars - groups treated with THIM. Asterisk indicates the significant difference.

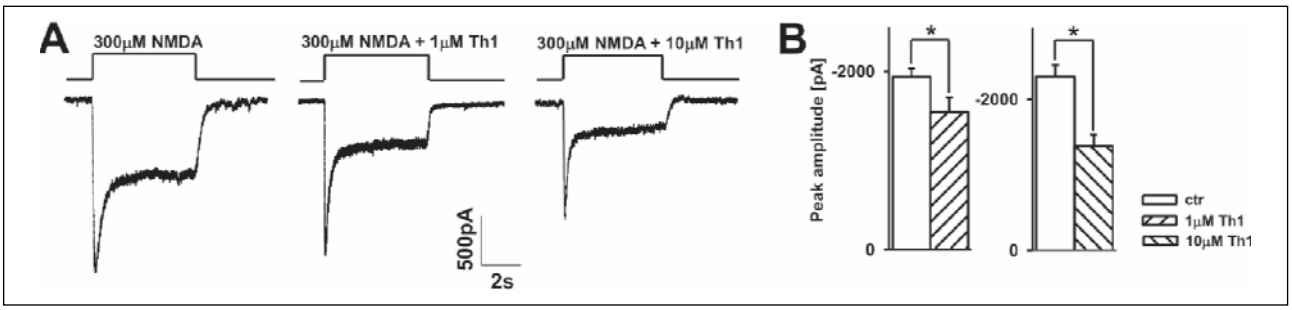

Fig. 3. Prolonged (60-90 min) treatment with THIM decreases the amplitude of NMDA-evoked currents. A, typical current responses elicited by rapid application of 300 μM NMDA in control conditions (left), in the presence of 1 μM thimerosal (middle) and in the presence of 10 μM thimerosal (right). Insets above the traces represent the time of agonist application. B, statistics of THIM effect on current amplitude at 1 μM (left) and at 10 μM (right). Note that at both THIM concentrations there were significant differences with respect to the controls. White bars represent controls white dashed bars - groups treated with THIM. Asterisks indicate the significant difference.

concentration (30 μM), there was no significant effect either (data not shown). Routine analysis of the current time course (10-90% rise time and deactivation kinetics) did not reveal any significant effect of THIM.

A similar series of experiments was carried out on the NMDA-evoked currents. A typical current evoked by a rapid application of 300 μM NMDA is shown in *Fig. 1C*. Addition of 100 μM THIM did not significantly affect the amplitude of NMDA-elicited currents, although a trend for current reduction was noted (relative amplitude 1.02±0.02, *Fig. 1D*, $p>0.05$). The impact of THIM (100 μM) was also tested at for responses elicited by a lower NMDA concentration (30 μM) but no significant THIM effect was observed (data not shown).

Prolonged treatment of neurons with THIM decreases GABAeric and NMDA-evoked currents

The experiments described above showed that THIM, following a brief exposure, did not exert any clear immediate effect either on GABA or NMDA-evoked currents. As shown in *Fig. 2A,B*, 60-90 min treatment of neurons with 1 μM THIM did not significantly affect the current elicited by 3 μM GABA (6.7% decrease; controls -1981.4±123.0 pA, n=46, for 1 μM THIM treatment -1848.1 pA±135.0 pA, n=42, $p>0.05$, *Fig. 2A,B*). However, when neurons were treated with 10 μM THIM, the amplitude of current response to 3 μM GABA was decreased by 23.4% (controls -1448.1±113.6 pA, n=51, for 10 μM THIM

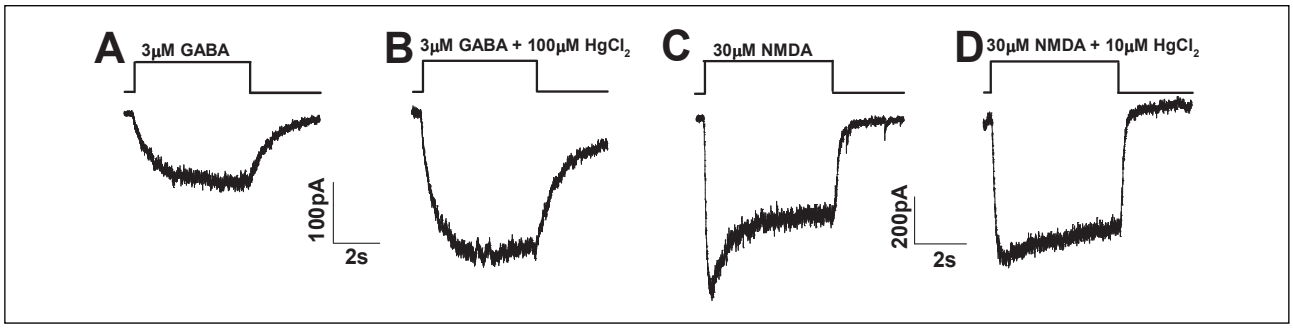

Fig. 4. Exemplary traces of current responses evoked by 3 μM GABA (5 s application) in control conditions (A) and in the presence of 100 μM of $HgCl_2$ (B). In C, typical control current evoked by 30 μM NMDA is shown and in D, current recorded from the same cell in the presence of 10 μM $HgCl_2$ is presented.

treatment -1101.5 pA±125.0 pA, n=40, $p<0.05$, *Fig. 2A,B*). As explained in Methods, in a single experiment, data for only one THIM concentration could be confronted with respective controls and therefore controls for 1 and 10 μM THIM were collected separately (in parallel to data obtained from THIM treated neurons). Similar experiments were carried out to examine THIM's effect on currents elicited by 300 μM NMDA (*Fig. 3*). In this case, both at 1 and at 10 μM THIM concentrations, a significant current decrease was observed by 20 and 40%, respectively (controls collected in parallel to treatment with 1 μM THIM treatment -1936.1±97.0 pA, n=43, for 1 μM THIM treatment -1543.28 pA±164.24 pA, n=43; controls collected in parallel to treatment with 10 μM THIM -2296.3±160.0 pA, n=42, for 10 μM THIM treatment -1377.71 pA±155.34 pA, n=41, *Fig. 3A,B*; $p<0.05$ for both THIM concentrations). These data indicate that prolonged exposure of neurons to low micromolar THIM concentrations significantly reduces both GABAergic and NMDA-evoked currents.

It needs to be stressed that the patch-clamp recordings are carried out on single neurons, whose conditions can be visually assessed at a standard microscope and always, the best looking neurons were selected. Nevertheless, because THIM is a known neurotoxin (5, 12, 27) longer neurons exposure to this compound could influence their viability and to test this possibility, standard trypan blue probe was applied (see Methods). We found that in control cultures, after 60-90 min exposure to the normal external solution, 18±2.5% of neurons (n=6 culture dishes tested) were classified as dead. Treatment with THIM for 60-90 min significantly increased the percentage of dead cells to 34±2.6 % for 1 μM THIM (n=6) and to 47±2.8% for 10 μM (n=6). Importantly, most neurons classified as dead based on the trypan blue test could be easily recognized visually as such and therefore the electrophysiological recordings would not be performed on such cells. Altogether, the trypan blue test showed that THIM is highly neurotoxic at low micromolar concentrations.

Effect of mercuric ions on GABAergic and NMDA-evoked currents

Since THIM molecule contains a mercury atom, we were curious how these ions had affected GABA- and NMDA-evoked currents. Previously, Huang and Narahashi (11) have shown that $HgCl_2$, at micromolar concentrations, significantly augmented GABA-induced currents in cultured neurons from rat dorsal root ganglia. In our experiments, 100 μM $HgCl_2$ was very toxic to hippocampal neurons. These cells showed visible signs of damage after only a few minutes of exposure, which was accompanied by a substantial rundown of GABA-evoked currents. For this reason a vast majority of recorded cells was not included in the analysis. In those sturdy cells, in which control recordings before application of $HgCl_2$ and after its washout were comparable, 100 μM $HgCl_2$ increased the current response to 3 μM GABA by nearly 120% (*Fig. 4A,B*), while at 10 μM concentration it increased these currents by approximately 20% (data not shown) that is in qualitative agreement with data reported by Huang nad Narahashi (11).

Analogously, the effect of $HgCl_2$ was tested on currents elicited by 30 μM NMDA. Again, the presence of this mercurial, especially at concentrations above 1 μM, resulted in clear deterioration of neurons' condition which was accompanied by a strong rundown of NMDA-evoked currents in the majority of cells from which recordings were made. At 1 μM concentration, $HgCl_2$ was ineffective in altering the NMDA-induced currents (relative amplitude 0.97±0.06, n=4, $p>0.05$), while at 10 μM - a moderate, but significant reduction of current amplitude was observed (0.76±0.05, n=7, $p<0.05$; *Fig. 4C,D*). Treatment with $HgCl_2$ resulted in a diminution of the fading phase of NMDA-elicited current suggesting the interference with the receptor kinetics. This effect was usually accompanied by a deterioration of patch and increase in baseline noise (data not shown).

DISCUSSION

In the present study we examined, using the patch-clamp technique in the whole-cell configuration, direct interactions of THIM with the $GABA_A$ and NMDA receptors in hippocampal neurons, following a brief (3-10 min) or prolonged (60-90 min) cell exposure to this drug. In addition, we compared THIM's effects to those of the inorganic mercurial, $HgCl_2$. To the best of our knowledge, this is the first report of THIM's interactions with these two receptors. Our results revealed that brief exposure to micromolar THIM concentrations did not cause statistically significant changes in GABA- and NMDA-induced currents, but 60-90 min treatment led to a significant decline of both types of currents. In contrast, a few minutes exposure of neurons to micromolar $HgCl_2$ caused marked reduction of the NMDA responses and increase of GABA-evoked currents. In the present study we have used a micromolar doses of THIM that probably exceed those in patients' brains (approximately 100 nM, 8). It needs to be emphasized, however, that mercurials supplied with vaccines accumulate in the brain tissue for several days (8) and it is likely that the effect reported here could appear at submicromolar THIM concentrations if administered for a prolonged time duration.

The modification of GABAergic currents by THIM and $HgCl_2$ resembles the effects reported previously for MeHg and $HgCl_2$ in dorsal root ganglia neurons, where inorganic mercurial

enhanced, while MeHg reduced these currents (11). The rapid effect of mercuric ions increasing the GABAergic currents is also consistent with the results of a neurochemical study, showing augmented benzodiazepine binding to the $GABA_A$ receptors in cerebellar neurons after incubation with micromolar concentrations of $HgCl_2$ and MeHg (10). However, in a functional biochemical study, incubation of microsacs from brain membranes with inorganic and organic mercurials ($HgCl_2$, hydroxymercuribenzoate or chloromercuriphenylsulfonic acid) attenuated GABA-stimulated chloride uptake (2). These ambiguous results suggest possible involvement of intermingled modulatory and toxic mechanisms. Mercurials most likely interact with cysteinyl -SH residues at the $GABA_A$ receptor complex (10, 11), critical for its gating properties (18, 26). Distinct electrophysiological effects of mercuric ions and THIM (or MeHg) on GABAergic currents is intriguing. It might potentially be due to dissimilar molecular state of Hg in these compounds and/or to its different accessibility to various functional -SH groups of the receptor complex. Clearly, the ionic form of Hg more readily reacts with external -SH groups than covalently bound Hg in the THIM molecule, but organic mercurials may more easily penetrate the lipid bilayer to get access to intracellular receptor domains. This may result in the distinct electrophysiological effects of $HgCl_2$ and THIM (or MeHg). Nonetheless, because THIM is metabolized in the body to ethyl mercury and subsequently to inorganic mercurials (22), which accumulates in the brain and other organs (8, 21), the delayed actions of THIM *in vivo* may be similar to those of mercuric ions. Furthermore, the potential modulatory effects of mercurials on the $GABA_A$ receptors will be - without any doubt - interwoven with their toxic effects on many cellular processes.

Inhibition of the NMDA-induced currents by THIM or other organomercurial has never been described before. Szucs *et al*. (25) have found that MeHg and $HgCl_2$ affected voltage-activated calcium channels, but appeared to have no clear direct effect on NMDA-evoked currents in rat cultured hippocampal neurons, except for a delayed action linked to the general toxicity of both mercurials. However, neurochemical studies conducted both *in vitro* and *in vivo* documented that $HgCl_2$ and MeHg at low micromolar concentrations inhibited ligand binding to the NMDA receptors in rat and mink brain (6, 23). Our findings of THIM's and mercuric ion ability to reduce the NMDA-induced currents are consistent with the latter studies and suggest some type of modulation. Most likely it involves interaction with the cysteine -SH groups, crucial for regulation of the NMDA receptor function, particularly for its redox-sensing properties (13, 15, 17). In living cells *in vitro*, these modulatory actions of mercurials are intermingled with their nonspecific neurotoxic effects, which are impossible to dissociate.

In conclusion, THIM and mercuric ions influence neurotransmission by interacting with $GABA_A$ and NMDA receptor complexes in live neurons in a complex manner. Mercuric chloride acts rapidly, decreasing electrophysiological responses to NMDA but augmenting responses to GABA, while thimerosal works slowly, reducing both NMDA and GABA responses. These modulatory effects - which most likely involve binding with functional thiol residues - are interwoven with neurotoxic actions of both mercurials.

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Author's address: Dr. Paulina Wyrembek, Laboratory of Neuroscience, Department of Biophysics, Wroclaw Medical University, 3 Chalubinskiego Street, 50-367 Wroclaw, Poland; Phone: +48 71 7841551; Fax: +48 71 7841399; E-mail: paulina_wyrembek@op.pl